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Variability in storage response within a coffee (*Coffea* spp.) core collection under slow growth conditions

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Abstract. An *in vitro* core collection of African coffee germplasm, structured in 32 diploid diversity groups, was established and conserved under slow growth for 3 years (6 subcultures). The initial objective was to store twenty accessions per group, with four replicates per accession. A statistical model was developed to analyse observations of survival rates within each diversity group. The goodness of fit of the model was shown. Survival analysis indicated a broad variability of the accessions in their response to the storage conditions and confirmed the importance of structuring the coffee complex down to the intraspecific level. Intra- and inter-group differences had consequences on the genetic representativity of the *in vitro* core collection. For practical purposes, conservation was carried on when the intra-group genetic drift was less than 50%.

Key words: *Coffea* - *in vitro* storage - survival model - genetic resources

Abbreviations: BA 6-benzyladenine, CAR Central African Republic, CIRAD Centre de Coopération Internationale en Recherche Agronomique pour le Développement, FAO Food and Agriculture Organization, IBPGR International Board for Plant Genetic Resources, IDEFOR-DCC Institut Des Fôrets - Département Café Cacao, ORSTOM Institut français de recherche scientifique pour le développement en coopération

Introduction

Since 1966, more than 10,000 wild African diploid coffee genotypes have been collected by ORSTOM, in collaboration with CIRAD, IBPGR and FAO in 7 African countries: Guinea and Côte-d'Ivoire in West Africa, Congo, Central African Republic and Cameroon in Central Africa, Kenya, and Tanzania in East Africa (Berthaud and Charrier 1988). Two base field collections were established in Côte-d'Ivoire (IDEFOR-DCC, Divo, and ORSTOM, Man) to conserve the germplasm collected. However, significant problems appeared with the maintenance in field genebanks: i) genetic erosion in some species due to their poor adaptation to the local environment and to attacks by pests and pathogens; ii) important labour costs and large space requirements. Thus, research for alternative methods to field conservation for coffee genetic resources became a priority (Berthaud and Charrier 1988).

Though coffee seeds can withstand desiccation down to 6-8 % water content (fresh weight basis), they cannot be considered orthodox because they remain cold-sensitive and desiccation does not improve their longevity (Van der Vossen 1977; Becwar et al. 1983; Ellis et al. 1990). When storing fully hydrated seeds at 19°C and under 100% relative humidity, the maximal storage duration obtained was 36 months for *Coffea arabica*, and 15 months for *C. canephora* and *C. stenophylla* (Couturon 1980). Thus, *in vitro* culture techniques appear to be the only alternative solution to field and *in situ* conservation for the safe conservation of coffee germplasm (Withers et al. 1990; Engelmann 1991).

Preliminary *in vitro* medium-term conservation experiments performed with shoots of several genotypes of *C. congensis*, *C. canephora*, *C. liberica* and *C. racemosa*, led to the selection of slow growth conditions, characterised by the very low BA (6-benzyladenine) content of the culture medium and subculturing intervals of 6 months (Bertrand-Desbrunais et al. 1991). This method allowed to store in our Laboratory the genotypes studied for seven years without any loss.

The establishment of an *in vitro* coffee core collection was then initiated in 1991. The main objective was to set up a small *in vitro* collection genetically representative of the large field genebanks conserved in Côte-d'Ivoire. The C strategy (Brown 1989) was adopted: 32 diploid diversity groups, representing 20 taxa of the sub-genus *Coffea*, were defined based on the available knowledge of the coffee complex genetic structure (Hamon et al. 1995). The objective was to conserve *in vitro* twenty accessions, with four replicates per accession, for each of the 32 groups defined.

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To be considered efficient, the genetic drift within each diversity group has to be controlled and the storage conditions should not lead to a progressive selection of the genotypes adapted to these conditions. This condition is particularly important when genes, rather than genotypes/clones, are conserved, as it is the case for the coffee core collection. In this study, the possibility of using the culture initiation method and the slow growth conditions defined by Bertrand-Desbrunais and Charrier (1989) and by Bertrand-Desbrunais et al. (1991), respectively, for the maintenance of a multispecific coffee collection was experimented and individual observations on each of the 620 accessions conserved *in vitro* were performed to assess the genotypic and diversity group response.

Table 1. Diversity Area, country of origin, year of introduction and number of accessions N maintained in April 1995 for the 32 diversity groups included in the *in vitro* coffee core collection.

Diversity group	Country of origin	Introduction year	N
C. brevipes Kumba Loum	Cameroon	1992-93	19
C. brevipes Mt Cameroon	Cameroon	1993	13
C. brevipes var. *heterocalyx*	Unknown	1993	8
C. canephora Cameroonese	Cameroon	1992	3
C. canephora Congolian	Congo, CAR	1992	1
C. canephora Guinean	Côte-d'Ivoire, Guinea	1992	2
C. canephora Nana	CAR	1992	0
C. congensis Cameroonese	Cameroon	1992-93	13
C. congensis Congolian	Congo	1992	7
C. congensis Central African	CAR	1992-93	18
C. costatifructa	Tanzania	1992	16
C. eugenioides	Kenya	1993	0
C. humilis	Côte-d'Ivoire, Guinea	1992-93	11
C. liberica Congolian	Cameroon, Congo, CAR	1992	18
C. liberica Guinean	Côte-d'Ivoire, Guinea	1992	18
C. liberica Koto	Cameroon	1992	19
C. pseudozanguebariae	Kenya, Tanzania	1993	5
C. racemosa Brazil	Mozambica	1992-93	18
C. racemosa Tanzania	Mozambica	1993	7
C. salvatrix Brazil	Mozambica	1993	3
C. salvatrix Tanzania	Mozambica	1993	2
C. sessiliflora Kitulangalo	Tanzania	1993	7
C. sessiliflora Shimba	Kenya	1992-93	20
C. sp. Congo	Congo	1992-93	7
C. sp. Mayombe	Congo	1993	7
C. sp. Moloundou Souanké	Congo	1992	15
*C.sp.*Moloundou Cameroon	Cameroon	1992-93	5
C. sp. Ngongo 2	Congo	1993	14
C. sp. Ngongo 3	Congo	1993	6
C. sp. Nkoumbala	Cameroon	1992	4
C. stenophylla East	Côte-d'Ivoire	1992	18
C. stenophylla West	Côte-d'Ivoire, Guinea	1992-93	8
Total			312

Materials and methods

Plant material. The geographical origin and the introduction years of the 32 diversity groups defined for developing the *in vitro* core collection are presented in Table 1. For each diploid diversity group, seeds bulks were provided from the base collections in Côte-d'Ivoire (IDEFOR-DCC, Divo and ORSTOM, Man), by harvesting seeds on randomly chosen trees. In order to minimise the risk of sampling inter-group hybrids, trees of the field border (5 trees) were not taken in account for bulking. Seeds were provided in 1992 for 21 diversity groups and in 1993 for the 11 other groups (Table 1). Diversity groups for which coffee-trees provided very few seeds in 1992 and those for which some disinfection problems occurred, were re-introduced in 1993 (Table 1).

Culture initiation. Before disinfecting, the testa was removed and seeds were washed with soap and tap water. Disinfection was achieved by soaking seeds in sodium hypochlorite (12%) for 15 min with continuous agitation on a rotary shaker, followed by 5 min under vacuum and 10 min again with agitation. After rinsing three times with sterile water, seeds were immersed for two days in sterile water for rehydration. Zygotic embryos were extracted from rehydrated seeds and inoculated on the germination medium (20 ml) defined by Bertrand-Desbrunais and Charrier (1989) in test tubes (250 x 24 mm) sealed with Parafilm Ribbon. Cultures were kept at 27 ± 1°C and 55 ± 2% relative humidity. Cultures were maintained in the dark until the hypocotyl stood upright and were then transferred to light conditions (30 $\mu E.m^{-2}.s^{-1}$, 12h light/12h dark photoperiod). Twenty well-developed seedlings were randomly chosen within each group of diversity.

Storage. Transfer of the shoot tips (1-2 cm long) to the slow growth medium was performed when the first two pairs of seedling leaves were developed. During subsequent transfers, the maximal number of microcuttings transferred was fixed at 4 per accession. Terminal or axillary shoot tips were preferentially chosen for microcuttings, but, when less then 4 shoot tips were available, node cuttings were used. All microcuttings were 1-2 cm long (2-4 nodes). The storage medium was previously described by Bertrand-Desbrunais et al. (1991). The BA content of the storage medium was 1.3 µM. Cultures were kept under light (30 $\mu E.m^{-2}.s^{-1}$, 12h light/12h dark photoperiod) at 27 ± 1°C and 55 ± 2% relative humidity. Accessions from seeds inoculated in January-February 1992 were subcultured 6 times, every 6 months, from the date of their transfer onto storage medium (June-August 1992). For the second introduction (January-February 1993), microcuttings were subcultured 4 times from September 1993.

Observations. The germination rate (%) was calculated for each group by dividing the number of well-developed seedlings by the number of non-contamined zygotic embryos established *in vitro*. At each transfer to fresh storage medium, the status (maintained/lost) of each accession was noted. Subculture 0 corresponded to the subculture on the germination medium, while the storage medium was used for subcultures 1 to 6. An accession was considered maintained if at least one microcutting was alive at the end of each subculture. Survival rate of each diversity group at Transfer n (SR_n) was calculated by dividing the number of accessions maintained by the initial number of seedling apical tips transferred onto the storage medium.

Survival model. Survival analysis was carried out by considering the following probabilistic model which assumed three hypothesis:

i) within each diversity group, there was a proportion N, with $0 \le N \le 1$, of genotypes which were not adapted to the storage conditions and a proportion A=1-N of adapted genotypes. The proportion A of adapted genotypes to the storage conditions could be maintained for an infinite number of subcultures,

ii) at each subculture, the probability of a non-adapted genotype to be maintained was p, with $0 \le p < 1$,

iii) A and p were independent.

The second assumption implied that the proportion N_n of non-adapted genotypes which were maintained during the nth subculture was

$$N_n = pN_{n-1}$$

where N_{n-1} was the proportion of non-adapted genotypes maintained at subculture n-1.

Then

$$N_n = p^n N_0 = p^n N$$

since

$$N_0 = N$$

The survival rate SR_n of a diversity group at Transfer n is

$$SR_n = N_n + A$$

thus

$$SR_n = Np^n + A = (1 - A)p^n + A$$

The survival model $SR_n = (1 - A)p^n + A$ was tested using the least square regression as computed by the Simplex method (Nelder and Mead 1965). The proportion of variance explained, *R square*, and the residual variance σ_e^2 were used for evaluating the fit of the model. The survival model was applied to the 21 diversity groups introduced in 1992. The model could not be tested when survival rates of a group had no variance, i.e. all the genotypes were maintained during the 6 subcultures.

Results

Contamination rates were low (0-29%), except for *C. brevipes* Kumba Loum (54%) and *C. congensis* Cameroonese (67%), and the disinfection protocol was considered effective. Germination rate was satisfactory in all diversity groups (76-100%), except *C. congensis* Central African (37%) and *C. sp.* Congo (33%, Table 2).

There was no correlation between the germination rate and the final survival rate (R^2=0.0283, P=0.4655).

Table 2. Class, germination rate GR (%), proportion of adapted genotypes *A*, probability of non-adapted accessions to be maintained at each Transfer *p*, proportion of variance explained by the regression model R^2 and residual variance σ_e^2 for the 21 diversity groups introduced in 1992.

Class	Diversity group	GR	*A*	*p*	R^2	σ_e^2
1	*C. congensis* Cameroonese	100.	1.00	-	-	-
	C. congensis Central African	37.	1.00	-	-	-
	C. humilis	100.	1.00	-	-	-
2	*C. liberica* Koto	88.	0.81	0.31	0.956	0.000
	C. brevipes Kumba Loum	100.	0.80	0.61	0.926	0.000
	C. stenophylla East	92.	0.77	0.45	0.886	0.001
	C. liberica Congolian	100.	0.76	0.61	0.962	0.000
	C. liberica Guinean	100.	0.72	0.92	0.898	0.000
	C. sp. Congo	33.	0.63	0.61	0.926	0.001
	C. costatifructa	100.	0.56	0.69	0.979	0.001
	C. sp. Moloundou Souanké	91.	0.55	0.77	0.986	0.000
	C. sp. Nkoumbala	100.	0.54	0.49	0.913	0.003
3	*C. congensis* Congolian	100.	0.54	0.00	1.000	0.000
4	*C. stenophylla* West	100.	0.12	0.62	0.917	0.011
	C. racemosa Brazil	100.	0.10	0.89	0.918	0.003
	C. canephora Cameroonese	76.	0.03	0.72	0.915	0.011
	C. canephora Congolian	100.	0.02	0.46	0.991	0.001
	C. sessiliflora Shimba	100.	0.00	0.93	0.915	0.002
	C. canephora Guinean	87.	0.00	0.66	0.849	0.029
	C. canephora Nana	100.	0.00	0.42	0.869	0.029
	C. sp. Moloundou Cameroon	81.	0.00	0.66	0.928	0.011

The proportion of variance explained by the model was always very high (85-100 %), the residual variance very low (0.000-0.029) and the goodness of fit of the model was shown (Table 2). The proportion *A* of adapted accessions and the probability *p* of non-adapted accessions to be maintained at each subculture were significantly independent (P=0.3375, R^2=0.0576).

Four Classes of diversity groups were obtained by the analysis of survival rates (Table 2): i) Class 1 included three diversity groups presenting no genetic erosion after 6 subcultures. For these groups, the survival rates had no variance and the model was not tested; ii) Class 2 contained nine groups which displayed a high proportion *A* of adapted accessions (54-81 %) ; iii) the *C. congensis* Congolian group formed the Class 3 and differed from groups of Class 2 by the nil probability ($p = 0$) of non-adapted genotypes to be maintained, i.e. all the non-adapted genotypes were lost during the first subculture; iv) the eight diversity groups of Class 4 included a very low proportion of adapted genotypes (0-23%). The evolution of survival rates in Classes 1, 2, 3 and 4 are illustrated in Figure 1 which shows results obtained with *C. congensis* Cameroonese, *C. costatifructa*, *C. congensis* Congolian and *C. canephora* Congolian. These four groups were representative of the other groups of their Class.

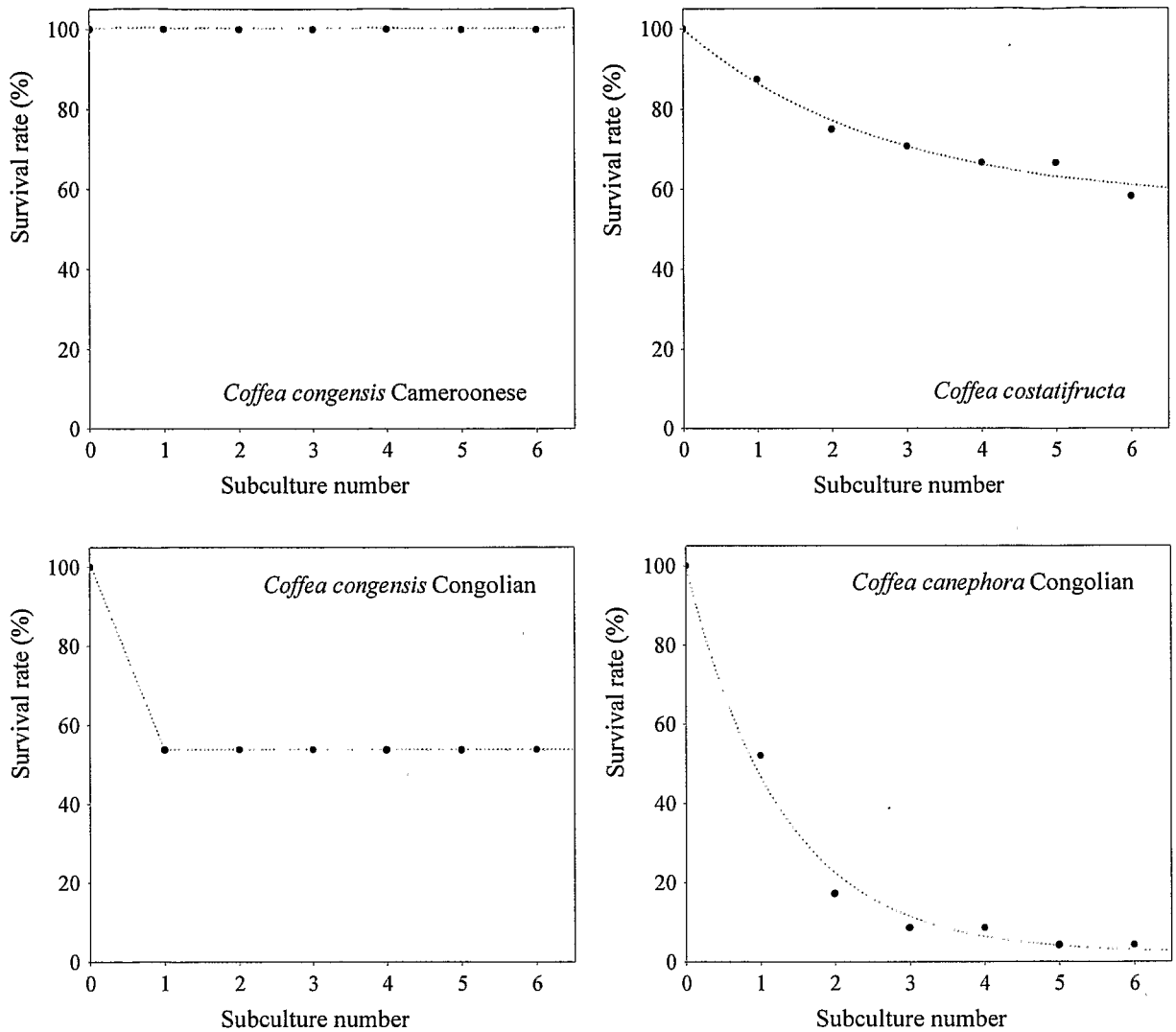
Groups defined within a same species could belong to different Classes, e.g. *C. congensis* Cameroonese and *C. congensis* Central African belonged to Class 1, whereas *C. congensis* Congolian was in Class 3; *C. stenophylla* West and *C. stenophylla* East were in Classes 2 and 4, respectively. Survival rates were not related to the geographical origin since each Class contained groups of different geographical origin, e.g. the countries of origin of groups of Class 1 were Cameroon, Central African and Côte-d'Ivoire/Guinea, but, Class 4 also contained groups from Cameroon (*C. canephora* Cameroonese, *C. sp.* Moloundou Cameroon), from Central African Republic (*C. canephora* Congolian, *C. canephora* Nana) and from Côte-d'Ivoire/Guinea (*C. stenophylla* West, *C. stenophylla* East and *C. canephora* Guinean). For groups which were introduced twice (Table 1), there was a significant correlation between the survival rate at subculture 3 of the two introductions (R^2=0.8464, P=0.0205).

Discussion

The goodness of fit of the model was shown for the 21 diversity groups tested and the estimated parameters, *A* and *p*, showed a great variability in response to the storage conditions, both at the intra- and the inter-group level. Differences in storage response between accessions have been reported notably for cassava (Roca et al. 1989), *Musa* (Van den Houwe et al. 1995), potato and sweet potato (Dodds 1989), *Xanthosoma* (Zandvoort et al. 1994) and yam (Malaurie et al. 1993). Besides, Henry et al. (1994) reviewed numerous studies which demonstrate that genetic factors condition *in vitro* tissue culture response, which could depend on genes involved in plant hormone metabolism. Thus, it appears that variability between accessions in their response to culture conditions is a general phenomenon. This implies that, where this phenomenon occurs, different sets of culture conditions have to be employed in the *in vitro* genebank.

In the present study, it was shown that genotypic selection occurred within coffee diversity groups of Classes 2, 3 and 4 and that this selection resulted in an

Figure 1. Observed survival rate (•, in %) from subculture 0 to subculture 6 and fitted survival evolution as computed by the Simplex method (dotted curve) of *Coffea congensis* Cameroonese, *C. costatifructa*, *C. congensis* Congolian and *C. canephora* Congolian microcuttings stored under slow growth conditions.



adaptation to storage conditions. For these groups, the adapted accessions maintained should not be considered as representative of the original group conserved in the field genebank. However, for practical purposes, it was considered that the representativity of a group was acceptable when the genotypic erosion was lower than 50% ($A>0.5$). Following this criterion, genetic groups of Classes 1, 2 and 3 could be stored under the conditions defined by Bertrand-Desbrunais et al. (1991). However, genetic representativity of the remaining genotypes should be verified using genetic markers. Molecular markers, such as RFLP and RAPD, have recently been developed on coffee (Lashermes et al. 1994 and 1993). Comparison of allelic frequencies between control (greenhouse-grown) and stored *in vitro* populations could be achieved. Larger effectives (e.g. n=100) should be introduced *in vitro* to allow a statistical comparison. Suitable mapped RFLP markers (Paillard et al. 1996) could be chosen so that most of the coffee genome would be covered.

For diversity groups of Class 4, especially for groups of the cultivated species *C. canephora*, further experiments should be performed to achieve higher final survival rates. Numerous parameters of the culture conditions which can lead to slow growth have been listed by Engelmann (1991) and could be tested in further experiments. The subculture interval could be optimized by monthly observations, as it is practised at CIAT for the cassava *in vitro* genebank (IPGRI/CIAT, 1994). Higher survival rates could also be achieved by manipulating, in combination, sucrose, BA, and mannitol contents and mineral strength in the culture medium. Bertrand-Desbrunais et al. (1991) showed, for *C. canephora*, that 0 and 4.4 µM BA concentrations were too low and too high respectively, but that intermediate BA levels should be tested. Even if reducing the sucrose concentration to 5 $g.l^{-1}$ had a negative effect on *C. arabica* microcuttings survival (Bertrand-Desbrunais et al. 1992), this factor could be

essential in *C. canephora* storage. The characteristics (height, axillary shoot development, rooting) of the microcuttings should also be evaluated at the beginning and the end of each subculture. This should allow an investigation of the influence and the interaction of the different factors tested on the two types of microcuttings (shoot tips and nodal cuttings). Jouve et al. (1992) observed that microcuttings of *C. canephora* were sensitive to storage at reduced temperature and this factor should not be tested. The use of mannitol, or any growth-retardant, should be considered with caution since it was shown that 100% of potato plants recovered from mannitol-induced slow growth displayed an hyper-methylation of DNA compared with controls (Harding, 1994).

Diversity groups belonging to the same species, e.g. *C. stenophylla* or *C. congensis*, displayed very different final survival rates (*A*). Therefore, this study confirmed the importance of structuring the coffee complex down to the intraspecific level, as suggested by Hamon et al. (1995). It was suggested by Bertrand-Desbrunais et al. (1991) that the specific response to slow growth conditions could be linked with the geographical origin. This hypothesis is not consistent with our results: e.g. though *C. humilis* and *C. canephora* Guinean groups were both collected in the same countries (Côte-d'Ivoire and Guinea), they presented very high and very low survival rates, respectively.

Our results indicate that viability evaluation has to be carried out when establishing an *in vitro* core-collection from non-identified material, i.e. group in which random sampling is performed to preserve a good representativity of the whole diversity. This does not apply to *in vitro* inoculation of clones from vegetative explants for which genotypic effect gives an all-or-nothing response. Moreover, the model proposed allows to calculate the number of seeds to inoculate to ensure that at least 20 accessions would be maintained in the core-collection, as determined by the binomial distribution and a chosen probability level.

The culture initiation protocol and the germination medium defined by Bertrand-Desbrunais and Charrier (1989) appeared appropriate for all the coffee taxa tested. This opens new perspectives for the distribution of coffee genetic resources in that exchange of *in vitro* seedlings should facilitate phytosanitary procedures and reduce the containment of plant material in quarantine (Withers 1987).

The initial objective of the present work was reached for numerous diversity groups since it was shown that it was feasible to preserve multispecific coffee genetic resources *in vitro*. In April 1995, 312 accessions, representing a very broad genetic diversity were conserved *in vitro* and 182 of them had been maintained for more than 3 years (Table 1). Long-term storage using cryopreservation should also be considered as a complementary option for the conservation of coffee germplasm. Previous studies have shown that seeds of *C. liberica* (Normah and Vengadasalam 1992) and zygotic embryos of *C. arabica* and *C. canephora* (Abdelnour-Esquivel et al. 1992) could withstand cryopreservation after partial desiccation. Additional experiments should be initiated to apply cryopreservation for the long-term conservation of all coffee species.

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